II. REMARKS

Formal Matters

Claims 8-12 and 23 are pending after entry of the amendments set forth herein.

Claims 8-12 were examined and were rejected.

Claim 8 is amended. The amendment to claim 18 was made solely in the interest of expediting prosecution, and is not to be construed as an acquiescence to any objection or rejection of any claim. The amendment to claim 18 was made to remove a redundancy. Accordingly, no new matter is added by the amendment to claim 18.

Claim 23 is added. Support for new claim 23 is found in the claims as originally filed, and throughout the specification, including the following exemplary locations: paragraph 0059; and original claims 8 and 11. Accordingly, no new matter is added by new claim 23.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

Rejection under 35 U.S.C.§102(b)

Claims 8-11 were rejected under 35 U.S.C.§102(b) as allegedly anticipated by Torrance et al. ((1998) *J. Biol. Chem.* 273:20810-20819; "Torrance").

The Office Action stated that Torrance discloses sequence-specific binding of Ku to single-stranded DNA; and that in the Materials and Methods "it is shown a nucleic acid sequence comprising a 5'TCG-3' sequence." Office Action, page 3. Applicants respectfully traverse the rejection.

Torrance indicates that the 5'-TCG-3' is not involved in Ku binding.

Torrance discusses single-stranded DNAse I footprinting analysis Jurkat nuclear factor binding to upper and lower strands of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) negative regulatory element 1 (NRE1). The lower strand had the sequence 5'-gtc**gtctttctctttctc**agtccggt-3' (with nucleotides corresponding to ⁻394 to ⁻381 shown in bold). Torrance states that the Jurkat nuclear extract protected nucleotides corresponding to NRE1 (⁻394/⁻381). Torrance, Figure 1A. The ⁻394/⁻381 sequence is 5'-gtctttctctttctc-3', which does not include 5'-tcg-3'. Torrance, page 20811, column 1, third paragraph under Materials and Methods. Thus, Jurkat nuclear factor binding site does not include the 5'-TCG-3' sequence.

Torrance used a single-stranded oligonucleotide having the sequence 5'-(actgagaaagagaaagacga)$_4$-3' in affinity chromatograph to bind factors in a Jurkat T cell nuclear extract. Torrance, page 20811, column 1, second paragraph under Materials and Methods. As the Office Action correctly noted, Ku was identified as a factor that bound the upper strand of NRE1. Torrance, page 20813, column 1, first and second full paragraphs. The Office Action failed to note, however, that the upper strand of NRE1 does not include a 5'-TCG-3' sequence. Furthermore, as shown in Figure 1B of Torrance, the portion of the upper strand that is protected in DNAse I footprinting experiments does not include the complement of the 5'-TCG-3' sequence, i.e., the sequence that is protected has the sequence 5'-gagaaagagaaagac-3', which would not include 5'-TCG-3' as a complementary sequence. Torrance, Figure 1B.

Torrance analyzed the sequence requirements for sequence-specific DNA binding by Ku, by comparing the binding of recombinant Ku to the upper strand of the GR MMTV LTR NRE1 with the binding of recombinant Ku to the polypurine-rich strands of various oligonucleotides. Torrance found that oligonucleotides that would not include 5'-TCG-3' in a complementary sequence do in fact bind Ku. Torrance, page 20815, Figures 5B and 5C. Furthermore, the HSE oligonucleotide, which would include 5'-TCG-3' in a complementary sequence, did not bind Ku. Torrance, Figures 5B and 5C. Thus, Torrance indicates that the 5'-TCG-3' sequence is not involved in Ku binding.

The fact that Torrance indicates that the 5'-TCG-3' sequence is not involved in Ku binding is supported by earlier work from the same laboratory. Giffin et al. ((1997) *J. Biol. Chem.* 272:5647-5658; "Giffin"; a copy of which is provided herewith as Exhibit 1). Giffin indicates that the binding site for Ku antigen in GR MMTV LTR is the overlapping 5'-gagaaga-3' sequence. Giffin, Figures 1, 3, and 4. Although a sequence complementary to the GR and C3H MMTV LTR includes 5'-TCG-3', sequences complementary to the c-myc PRE and U5 HTLV LTR sequences do not include 5'-TCG-3', yet the c-myc PRE and U5 HTLV LTR compete efficiently with the GR MMTV LTR for binding to Ku. Giffin, Figure 3. Furthermore, a sequence complementary to the HSE sequence does include 5'-TCG-3', yet the HSE does not bind Ku and does not compete with GR MMTV LTR for binding to Ku. Giffin, Figures 3 and 4.

Torrance does not disclose or suggest all the claim limitations.

Torrance neither discloses nor suggests a method for identifying an agent that modulates a biological activity of DNA-PK, wherein the method comprises adding an agent to be tested to a sample comprising DNA-PK and an immunomodulatory nucleic acid. Torrance analyzed binding of Ku antigen

to DNA. Torrance did not analyze a biological activity of DNA-PK. DNA-PK comprises Ku antigen and DNA-PK_{cs}. Torrance did not analyze any effects of an agent on a biological activity of DNA-PK.

Furthermore, as discussed above, Torrance does not disclose or suggest a method involving contacting a sample comprising DNA-PK and an immunomodulatory nucleic acid having a recited sequence. Torrance neither discloses nor suggests a method for identifying an agent that modulates a biological activity of DNA-PK, wherein the method comprises adding an agent to be tested to a sample, the sample comprising DNA-PK and an immunomodulatory nucleic acid molecule, under conditions which favor binding of the immunomodulatory nucleic acid molecule to DNA-PK, thereby forming a test sample, wherein the immunomodulatory nucleic acid molecule is a DNA molecule that, when bound to Ku antigen, activates DNA-PKcs, wherein the immunomodulatory nucleic acid molecule comprises a nucleotide sequence selected from 5'-Purine-Purine-C-G-Pyrimidine-Pyrimidine-3', 5'-Purine-TCG-Pyrimidine-Pyrimidine-3'; 5'-$(TCG)_n$-3', where n is any integer that is 1 or greater, 5'-Purine-Purine-CG-Pyrimidine-Pyrimidine-CG-3', 5'-Purine-TCG-Pyrimidine-Pyrimidine-CG-3', and 5'-Purine-Purine -CG-Pyrimidine-Pyrimidine-CG-3'. As discussed above, Torrance does not discuss use of nucleic acids having the recited sequences.

Furthermore, Torrance provides no disclosure or any indication whatsoever that the oligonucleotides used in the analysis of Ku binding are actually *immunomodulatory nucleic acids*.

Torrance does not disclose or suggest analyzing a biological activity of DNA-PK. Torrance does not disclose or suggest a method involving use of an immunomodulatory nucleic acid having a recited sequence. Therefore, Torrance does not disclose or suggest all of the claim limitations. Because Torrance does not disclose or suggest all of the claim limitations, Torrance cannot anticipate the instant invention as claimed.

Applicants submit that the rejection of claims 8-11 under 35 U.S.C. §102(b) has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

Rejection under 35 U.S.C.§103(a)

Claims 8-12 were rejected under 35 U.S.C.§103(a) as allegedly unpatentable over Torrance in view of Kelavkar et al. ((2000) *Genes and Immunity* 1:237-250; "Kelavkar").

The Office Action stated: 1) Torrance discloses sequence-specific binding of Ku to single-stranded DNA; and 2) Kelavkar discloses that Ku antigen is required for IL-13/4 induction of lipoxygenase-1 gene expression in human epithelial cells. The Office Action concluded that it would have been obvious at the time the invention was made to modulate DNA-PK activity by measuring an amount of IL-6 or IL-12 produced by the cell, since Kelavkar further discloses that the biological significance of Ku expression has also been studied in aging and in cancer. Applicants respectfully traverse the rejection.

As discussed above, Torrance does not disclose or suggest a method for identifying an agent that modulates a biological activity of DNA-PK. Torrance analyzed binding of Ku antigen (not DNA-PK) to various oligonucleotides. Torrance does not disclose or suggest a method involving adding a test agent to a sample comprising DNA-PK and an immunomodulatory nucleic acid having a recited sequence. The Office Action stated that one of the sequences in Materials and Methods includes a 5'-TCG-3' sequence. Torrance indicates that the sequence 5'-TCG-3' is not involved in Ku binding.

Kelavkar does not cure the deficiency of Torrance. Kelavkar merely reports that, in A549 cells, Ku antigen is induced in response to the cytokines IL-13 and IL-4. Torrance, alone or in combination with Kelavkar does not teach all of the claim limitations. The Office Action has not presented any reasoning as to any motivation to combine the references, or as to any expectation of success. Accordingly, the Office Action has not established a prima facie case of obviousness.

Applicants submit that the rejection of claims 8-12 under 35 U.S.C. §103(a) has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

III. CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number UCAL168.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: June 15, 2004

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Sequence-specific DNA Binding and Transcription Factor Phosphorylation by Ku Autoantigen/DNA-dependent Protein Kinase

PHOSPHORYLATION OF Ser-527 OF THE RAT GLUCOCORTICOID RECEPTOR*

(Received for publication, June 27, 1996, and in revised form, December 11, 1996)

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NRE1 is a DNA sequence element through which Ku antigen/DNA-dependent protein kinase (DNA-PK) catalytic subunit represses the induction of mouse mammary tumor virus transcription by glucocorticoids. Although Ku is an avid binder of DNA ends and has the ability to translocate along DNA, we report that direct sequence-specific Ku binding occurs with higher affinity (K_d = 0.84 ± 0.24 nM) than DNA end binding. Comparison of Ku binding to several sequences over which Ku can accumulate revealed two classes of sequence. Sequences with similarity to NRE1 competed efficiently for NRE1 binding. Conversely, sequences lacking similarity to NRE1 competed poorly for Ku and were not recognized in the absence of DNA ends. Phosphorylation of glucocorticoid receptor (GR) fusion proteins by DNA-PK reflected Ku DNA-binding preferences and demonstrated that co-localization of GR with DNA-PK on DNA in *cis* was critical for efficient phosphorylation. Phosphorylation of the GR fusion protein by DNA-PK mapped to a single site, Ser-527. This site occurs adjacent the GR nuclear localization sequence between the DNA and ligand binding domains of GR, and thus its phosphorylation, if confirmed, has the potential to affect receptor function *in vivo*.

DNA-dependent protein kinase (DNA-PK)[1] is a nuclear kinase with a unique catalytic requirement for direct DNA contact (1–4). Specific targeting of DNA-PK to DNA binding sites *in vivo* is likely to be critical for determining substrate phosphorylation for two reasons. First, the affinity of DNA-PK for substrate (K_m = 210 μM) is low. Second, DNA-PK is a broad specificity kinase with a very simple recognition sequence (Ser-Gln or Thr-Gln) (1, 2). Therefore, the obligate linking of DNA-PK to DNA is expected to favor phosphorylation of co-localized substrates in the cell nucleus.

Targeting of DNA-PK_{cs} to DNA is by Ku autoantigen (p70/p80) (4, 5), which is both a DNA-binding subunit of DNA-PK and an allosteric regulator of kinase activity (5). Ku was first identified as a factor stimulating autoantibody production in a variety of mixed connective tissue autoimmune diseases (6, 7). Ku is an avid, but unusual, DNA-binding protein. First demonstrated to bind DNA ends (7, 8), Ku is now suspected to recognize virtually any DNA structure containing a double- to single-stranded DNA transition (9). Remarkably, once bound to DNA, Ku is able to translocate from its point of entry (8). However, while Ku translocates freely along DNA *in vitro*, it appears to be unable to translocate through a nucleosome (10). Thus, the effective range of Ku translocation *in vivo* is likely to be quite limited.

$DNA\text{-}PK_{cs}$ has extensive similarity to the *Ataxia telangiectasia* gene product and to the family of lipid kinases that includes phosphatidylinositol 3-kinase (11–13). Recently, DNA-PK has been shown to play important roles in double-stranded DNA break repair and V(D)J recombination (14). Inactivation of $DNA\text{-}PK_{cs}$ or Ku-p80 results in overlapping, but distinct recombination and repair deficiencies (15–22). $DNA\text{-}PK_{cs}$ has been shown to be required for the resolution of coding ends during V(D)J recombination, and mutation in $DNA\text{-}PK_{cs}$ has been proposed to account for the defects in the severe combined immunodeficient (*SCID*) mouse. In addition to recruiting $DNA\text{-}PK_{cs}$ to repair and recombination sites, Ku is proposed to protect the ends of recombination and repair intermediates from exonuclease digestion (23).

The relaxed substrate specificity of DNA-PK *in vitro* has led to proposals for its participation in other nuclear processes, particularly transcription and DNA replication. For example, a role for DNA-PK in repressing RNA polymerase I transcription has been suggested (24–26). Additionally, a large number of sequence-specific transcription factors have been shown to be substrates for DNA-PK *in vitro* (1, 27, 28), suggesting a role in the regulation of transcription by RNA polymerase II. Indeed, DNA-PK has recently been shown to be associated with the RNA polymerase II holoenzyme (29).

As the targeting of Ku to transcriptional regulatory regions by translocation from DNA ends is likely to very limited, the

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¶ Recipient of a studentship from the National Sciences and Engineering Research Council of Canada.

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[1] The abbreviations used are: DNA-PK, DNA-dependent protein kinase; $DNA\text{-}PK_{cs}$, DNA-dependent protein kinase catalytic subunit; NRE1, negative regulatory element 1; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; GR, glucocorticoid receptor; Oct-1, octamer transcription factor 1; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; HTLV, human T cell leukemia virus; IAP, intracisternal A particle; PRE, plasmacytoma repressor factor binding site; DBD, DNA binding domain; GRE, glucocorticoid-responsive element; PSE, proximal U1 promoter sequence element; HSE, heat shock response element; bp, base pair(s); HPLC, high pressure liquid chromatography; Ab, antibody.

potential ability of Ku to bind directly to transcriptional regulatory elements has generated considerable interest. Over the past several years, there have been numerous reports of the accumulation of Ku *in vitro* over DNA sequence elements that function to regulate transcription (9, 30–37). It has not been clear, however, whether the presence of Ku over these sequences represented direct sequence-specific DNA recognition or the accumulation of Ku translocated from the ends of the DNA fragments employed.

Recently, we demonstrated that a DNA sequence element, NRE1 (negative regulatory element 1), in the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) is a direct, sequence-specific, DNA binding site for Ku/DNA-PK$_{cs}$ in the absence of DNA ends or other structural features (38). In transfection experiments, NRE1 specifically repressed glucocorticoid hormone activated MMTV transcription in a Ku- and DNA-PK$_{cs}$-dependent manner. Further, *in vitro*, DNA-PK efficiently directed the phosphorylation of glucocorticoid receptor (GR) and octamer transcription factor 1 (Oct-1), two transcription factors that bind the MMTV promoter *in vivo* in response to glucocorticoids (39–41).

In the experiments presented here, we have examined the DNA requirements for Ku binding and DNA-PK phosphorylation of rat GR in detail. Our results demonstrate that sequence-specific Ku binding to NRE1 is strongly preferred to DNA end binding and that DNA-PK catalytic activity closely parallels Ku DNA-binding preferences. Furthermore, our data indicate that only the subset of previously proposed sequence-specific Ku binding sites with similarity to NRE1 are likely to be direct sequence-specific Ku binding sites. Experiments with a GR fusion protein substrate indicated that linkage of DNA-PK and substrate in *cis* on DNA markedly increased the efficiency of substrate phosphorylation. Intriguingly, phosphorylation of the GR fusion protein occurred at Ser-527, in the region of the receptor connecting the DNA and ligand binding domains and immediately adjacent to the dominant nuclear localization sequence of the receptor.

MATERIALS AND METHODS

Oligonucleotides and Microcircles—Oligonucleotides were synthesized on a Beckman Oligo 1000 DNA synthesizer. Sequences of the upper-strands of oligonucleotides are shown in Fig. 1, except for the oligonucleotide containing a binding site for nuclear factor 1 (NF1), upper strand (5′-GTTCTTTTGGAATCTATCCAAGTCTTA-3′). The NRE1 and PRE oligonucleotides have been described previously (42). Microcircles containing the GR- or C3H-MMTV NRE1 elements, four copies of a consensus octamer motifs, the U1 small nuclear RNA PSE, rc-*mos* IAP enhancer core or HSE were prepared as described previously from 223- or 292-bp pBluescript fragments containing one (NRE1, U1, IAP, HSE) or four copies (octamer) of the individual oligonucleotides cloned into the *Sma*I site of pBluescript (38). Purified recircularized DNA was completely resistant to S1 nuclease, exonuclease III, and Bal31.

Plasmids and Recombinant Proteins—Expression and purification of Ku from insect cells was essentially by the protocol of Ono *et al.* (43). Baculovirus expression vectors VBB2-Kup86 and VBB2-Kup70tH^6, encoding the p86 subunit and a hexahistidine-tagged p70 subunit of human Ku autoantigen, respectively, were co-infected into Sf9 cells. Three days post-infection, cells were harvested and lysed by sonication in 40 mM HEPES, pH 7.9, 1 mM EDTA, 2 mM dithiothreitol, 0.1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Ku heterodimers were then purified using a Ni^{2+} affinity resin (His-Bind Resin, Novagen).

pGEX-2T-X568 encoding glutathione *S*-transferase GR fusion protein containing amino acids 407–568 of rat GR (GST-GR) was created by cloning the *Bam*HI-*Eco*RI fragment of pSP64X568 (44) in frame into the *Bam*HI-*Eco*RI sites of pGEX-2T (Pharmacia). pGEX-2T-C500Y encoding GST-GR containing a Cys → Tyr mutation at position 500 of the DBD (GST-GR$_{C500Y}$) was created by introduction of a *Bam*HI-*Sma*I fragment of pT7C500Y (45) in frame into the *Bam*HI-*Sma*I sites of pGEX-2T. GST fusion proteins were expressed in *E. coli* by growing transformed cells at 37 °C overnight, followed by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4–16 h at 23 °C. Induced bacteria were harvested and resuspended in lysis buffer (25 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) supplemented with 100 μg/ml lysozyme and incubated for 10 min at 4 °C. Cells and DNA were sheared by passage through a series of 18-, 20-, and 25-gauge needles, followed by sonication 10 times for 40 s in the presence of 0.1% Nonidet P-40 at 4 °C. Lysates were centrifuged 10 min at 10,000 × *g*, and the supernatants were incubated with 3 ml of glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.) for 90 min. The beads were subsequently washed five times with 10 bed volumes of lysis buffer and three times with 20 bed volumes of 60% lysis buffer containing 0.1% Nonidet P-40. GST-GR beads were stored at 4 °C with 1 mM phenylmethylsulfonyl fluoride and 0.02% NaN_3. To separate the GST domain from the DBD, 20 μl of a 1:1 slurry of beads were incubated with 0.5 units of thrombin (Novagen) at room temperature for 30 min.

pHC17 and pHC364 have been described previously (46). Linear and covalently closed circular plasmids were prepared as described previously (38). Covalently closed circular plasmids were completely resistant to digestion with exonuclease III, Bal31, and S1 nuclease.

Electrophoretic Mobility Shift Assays (EMSAs)—Binding of purified baculoviral Ku to 10.8 pmol of ^{32}P-kinased microcircle DNA was performed in 20 μl of 12 mM HEPES, pH 7.9, 12% glycerol, 60 mM KCl, 0.12 mM EDTA, 1 μg of BSA. 1 μg of highly sheared calf thymus DNA was included in assays with Jurkat nuclear extracts. Binding reactions were incubated for 20 min at room temperature. Monoclonal antibodies included in mobility shift incubations included Ku antibody 162 (47), octamer transcription factor-homeodomain antibody YL 15 (48), and GR antibody BuGR (49). For competition experiments, increasing concentrations of unlabeled oligonucleotides were added with the ^{32}P-labeled GR-MMTV NRE1 microcircle to the binding mix prior to addition of Ku. Protein-DNA complexes were resolved by electrophoresis on 3% polyacrylamide gels (acrylamide/bisacrylamide = 30:1) in 0.5 × TBE for 300 V-h. Gels were dried and exposed to autoradiography film (DuPont) using Reflection intensifying screens (DuPont) and/or quantified by phosphorimage analysis using a Bio-Rad GS-525 Molecular Imager System.

The dissociation constant (K_d) of baculovirus-expressed Ku for NRE1 containing microcircles was determined in a manner similar to that described previously (50–52). EMSA was performed with a constant amount of recombinant Ku incubated with an increasing concentration of DNA (2.7–43.5 pmol) in a 40-μl reaction volume. After electrophoresis the bound and free DNA were quantified by phosphorimager. To determine the concentration of bound DNA, a standard curve was created using the total (bound + free) adjusted volume (counts × mm^2) from the phosphorimager *versus* concentration of total DNA. Total DNA concentration was determined by subsequent scintillation counting of excised bands (Beckman LS-3801). Labeling efficiency was 100%, as formation of covalently closed microcircles required incorporation of [α-^{32}P]dATP during fill in of the *Xho*I site prior to microcircle ligation. K_d was determined by Scatchard analysis (K_d = −1/slope) in three independent trials and expressed as mean ± S.E.

Phosphorylation of GST-GR by DNA-PK—Phosphorylation of GST-GR by DNA-PK was performed essentially as described previously (38) using 10 ng of supercoiled, linear, or covalently closed circular pHC17 or pHC364. Plasmids were linearized with *Hin*dIII as indicated and, in some instances, recircularized with T4 DNA ligase. Supercoiled, covalently closed circular, and linear plasmids were purified through agarose gels prior to use in kinase assays. Kinase reactions were carried out for 15 min at 30 °C in kinase buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM EGTA) using 10 ng of plasmid DNA, 0.5 units of DNA-PK (Promega), 5 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, DuPont), and 5–15 μl of a 1:1 slurry of GST-GR bound to glutathione-Sepharose beads, prewashed twice in kinase buffer. Following kinasing, the beads were washed three times in kinase buffer to remove free [γ-^{32}P]ATP. The GST-GR was eluted by boiling in SDS-PAGE sample buffer prior to or following thrombin cleavage as indicated. ^{32}P incorporation was determined by autoradiography or phosphorimage analysis of 12% SDS-PAGE gels.

Identification of DNA-PK Phosphorylation Sites on GST-GR—Phosphorylated GST-GR was recovered from SDS-PAGE gels, digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, and analyzed by reverse-phase HPLC and electrophoresis on 40% alkaline polyacrylamide gels as described previously (53). Prior to further manipulation, tryptic peptides were eluted from alkaline polyacrylamide gels, dried, redissolved in 0.1% trifluoroacetic acid, and purified over SepPak C18 columns (Millipore).

Digestion of purified phosphopeptide with sequencing grade endo-

FIG. 1. **Alignment of several sequences with the potential for sequence-specific Ku binding suggests two categories of motif.** *A*, alignment of potential sequence-specific Ku binding sites with extensive similarity to NRE1. Regulatory elements with homology to the GR-MMTV NRE1 element (38, 42) include the C3H element (59), the PRE upstream of the c-*myc* gene (57), and a transcriptional repressor element in the U5 HTLV LTR (36). Matches with NRE1 are *uppercase*, mismatches *lowercase*. An overlapping direct repeat within the GR-MMTV NRE1 core is highlighted by *arrows*. *B*, potential sequence-specific Ku binding sites lacking obvious homology to NRE1. Proposed Ku binding sites lacking homology to NRE1 include the octamer motif binding site (34) of octamer transcription factors (octamer motif *underlined*), the PSE from the U1 promoter (32), a heat shock response element (31), and an enhancer core sequence from a recently transposed (rc-*mos*) IAP LTR (63), which was subsequently proposed to contain a Ku binding site (9).

proteinase AspN (Boehringer Manheim) was performed using 0.2 μg of AspN (54) in 200 μl of 50 mM sodium phosphate buffer, pH 8.0, for 8–16 h at 37 °C. Additional aliquots of AspN were added at 4-h intervals. Digestion products were resolved on 40% alkaline polyacrylamide gels (55).

Manual [^{32}P]phosphate release studies were performed on purified peptide according to a protocol originally described by Sullivan and Wong (56), as modified by Zhang *et al.* (53). Phenylisothiocyanate was obtained from J. T. Baker Inc. Triethylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and trifluoroacetic acid were obtained from Sigma. Sequelon-AA membrane was obtained from Millipore Corp. (Milford, MA).

RESULTS

Specificity of DNA Recognition by Ku Autoantigen—Recently, we demonstrated that Ku antigen bound on fully relaxed, covalently closed circular DNA, to a region of the LTR from the GR strain of MMTV (−397 and −375) centered over NRE1 (38). Furthermore, in EMSA a 23-bp oligonucleotide spanning LTR sequences −398 and −376 was sufficient for Ku binding to a microcircle (38). Previously, a number of other potential sequence-specific Ku binding sites had been proposed (9, 24, 30–37). However, these experiments could not distinguish between direct sequence-specific binding of Ku and accumulation of Ku translocated from a DNA end.

Alignment of these proposed Ku binding sites with NRE1 revealed two types of DNA sequence, several examples of which are displayed in Fig. 1. First, regulatory elements flanking the murine c-*myc* gene (57, 58) and in the LTR of human T cell leukemia virus (HTLV) (36) have the same overall polypurine character as NRE1 and considerable sequence identity (Fig. 1*A*). In the C3H strain of MMTV, there is a similar conservation over NRE1 and this region of the C3H LTR has similarly been implicated in regulating viral transcription (59–61). A second category of potential sequence-specific Ku binding site is composed of sequences with no apparent homology to NRE1, or even to each other (Fig. 1*B*). This group of sequences includes the octamer motif bound by octamer transcription factors (34), a heat shock response element (31), an element from the U1 small nuclear NA promoter (32), and a high affinity EBP80 binding site from the rc-*mos* intracisternal A particle (IAP) LTR (9, 62, 63). The lack of similarity to NRE1, or indeed to each other, is clearly suggestive of an alternative mechanism for the recognition of these sequences by Ku. In order to begin to distinguish the requirements for Ku binding to different DNA sequences and structures, we compared the binding of Ku to NRE1 with Ku binding to NRE1-like sequences, unrelated sequences, and DNA ends.

The human Ku protein used in this study was prepared from baculovirus as described previously (43), with the cDNA for the p70 subunit encoding a histidine tag used to purify the Ku heterodimer by nickel affinity chromatography (Fig. 2*A*). This form of Ku recognizes NRE1 indistinguishably from human Ku purified from Jurkat T cells (38). In the first experiment (Fig. 2*B*), we compared Ku binding to the NRE1 elements in the LTRs of the GR- and C3H MMTV strains on covalently closed microcircles. The microcircles used in these assays were gel-purified and resistant to digestion with exonuclease III, Bal31, and S1 nuclease, ensuring the absence of nicks and structural features in the microcircles (38).[2] The C3H-NRE1 element has several differences in sequence from the GR element, but retains the overall polypurine character of GR-NRE1 (Fig. 1*A*). Incubation of microcircles containing either GR-NRE1 (Fig. 2, *B* and *C*, *lanes 1–4*) or C3H-NRE1 (Fig. 2*B*, *lanes 5–8*) with recombinant Ku expressed from baculovirus resulted in the formation of a protein-DNA complex in EMSA that was supershifted by an antibody to Ku, but unaffected by a glucocorticoid receptor antibody. No binding was detected to microcircles lacking an NRE1 insert.[2] However, over several repetitions of the binding assay, Ku binding to C3H-NRE1 microcircles was consistently about 4-fold lower than binding to the GR-NRE1 microcircles. Thus, while the sequence differences between the GR- and C3H-NRE1 elements do not prevent recognition by Ku, they do appear to influence the affinity of binding.

To compare direct Ku binding to NRE1 with binding to a proposed sequence-specific Ku binding site unrelated in sequence to NRE1, we evaluated the binding of Ku to microcircles containing octamer motifs (Fig. 2*C*). By contrast to NRE1 binding, recombinant Ku was unable to recognize microcircles containing four copies of an octamer motif oligonucleotide (Fig. 2*C*, *lane 6*). This finding was consistent with our previous result that the two octamer motifs in the MMTV LTR were unable to support DNA-PK activity in the absence of DNA ends (38). To confirm that Ku was unable to bind covalently closed circular DNA containing octamer motifs, we repeated the binding assay

[2] W. Giffin and R. J. G. Haché, data not shown.

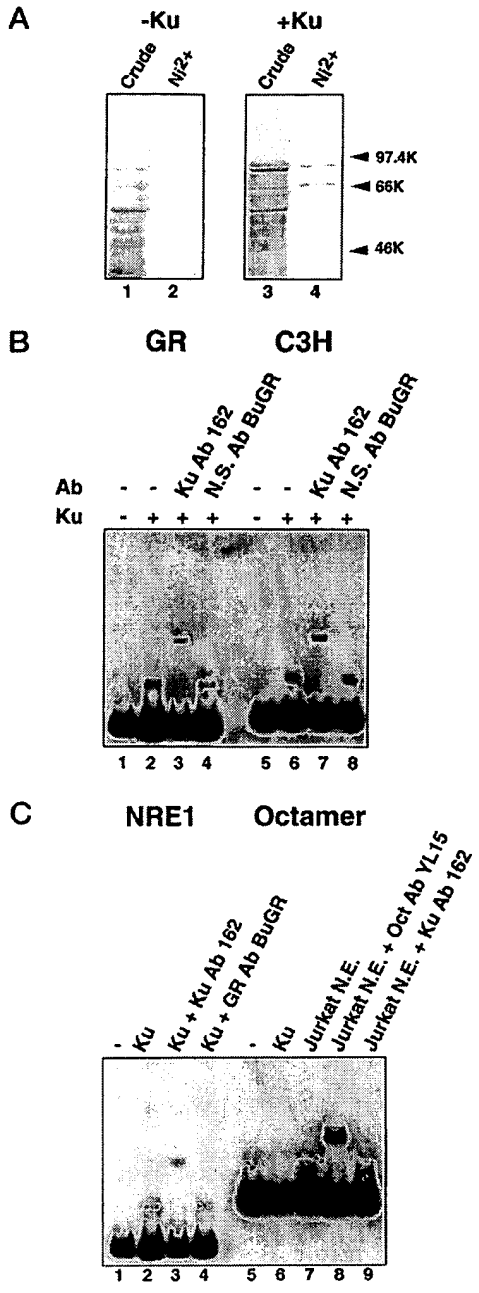

FIG. 2. **Ku binds specifically to the NRE1 element in of the C3H strain of MMTV, but is unable to bind an octamer motif.** *A*, silver-stained gel of recombinant Ku purified from baculovirus-infected insect cells by chromatography over a Ni^{2+} affinity column. *Lanes 1* and *3* show the pattern of staining in the crude extract obtained from mock-infected and Ku-infected Sf9 cells, respectively. *Lanes 2* and *4* show the Ni^{2+} affinity column eluates. *B*, EMSA comparison of recombinant Ku binding to GR (*lanes 1–4*) and C3H NRE1 sequences (*lanes 5–8*) on covalently closed circular microcircles resistant to exonuclease III, Bal31, and S1 nuclease. *XhoI/PvuII* microcircles of 223 bp (*lanes 1–4*) or 227 bp (*lanes 5–8*) prepared from pBluescript containing NRE1 oligonucleotides from the GR (*lanes 1–4*) and C3H (*lanes 5–8*) strains of MMTV were electrophoresed through a 3% polyacrylamide gel following incubation in the presence of 1 μg of highly sheared calf thymus DNA and in the presence or absence of recombinant Ku and/or specific and nonspecific antibodies as indicated. Ku Ab 162 is a monoclonal antibody (*Ab*) that supershifts Ku-DNA complexes, while nonspecific (*N.S.*) antibody BuGR is a glucocorticoid receptor monoclonal antibody that is unable to recognize Ku. The 1 μl of recombinant Ku added in the GR-NRE1 binding assays resulted in approximately 40% of the Ku-microcircle complexes obtained with 10 μl of Ku added to the C3H-NRE1 microcircle, as measured by phosphorimager. *C*, evaluation of Ku binding to a 223-bp microcircle containing the GR-MMTV NRE1 element (*lanes 1–4*) and binding to a 300-bp microcircle containing 4 copies of an oligonucleotide with a consensus octamer motif (*lanes 5–9*). *Lanes 1* and *5* show the results of control incubations in the absence of Ku and Jurkat nuclear extract. In *lanes 2–4* the NRE1-containing microcircle was incubated with 1 μl of recombinant Ku (*lane 2*) together with Ku Ab 162 (*lane 3*) or nonspecific antibody BuGR (*lane 4*). *Lanes 6–9* show the results of incubation of the octamer motif-containing microcircle with 10 μl of Ku (*lane 6*) or 1 μg of Jurkat nuclear extract (*N.E., lanes 7–9*) in the presence of Oct homeodomain antibody YL15 that supershifts octamer proteins bound to DNA (*lane 8*) or Ku Ab 162 (*lane 9*).

with nuclear extract prepared from Jurkat T cells, which contain an abundance of both octamer transcription factors and Ku (38, 64). Incubation of the octamer motif-containing microcircle with Jurkat nuclear extract led to the formation of a complex that was supershifted (Fig. 2*C*, *lanes 7* and *8*) by an antibody that is known to supershift octamer transcription factors (48). The Ku antibody was, however, completely unable to modify the shifted complex obtained with Jurkat extract (*lane 9*). Previously, we demonstrated that this antibody effectively supershifted Jurkat nuclear extract Ku binding to NRE1 (38). Therefore, we conclude that the C3H-MMTV NRE1-like sequence is, like its GR counterpart, a direct, internal, sequence-specific binding site for Ku antigen; however, a consensus octamer motif is not directly recognized by Ku.

In a second experiment, we compared the ability of oligonucleotides from the two categories of proposed Ku binding site, and completely nonspecific DNA, to compete binding of recombinant Ku to microcircles containing NRE1 from the GR strain of MMTV (Fig. 3). As direct sequence-specific Ku binding to NRE1 was resistant to a large excess of highly sheared calf thymus DNA (Fig. 2), we anticipated that oligonucleotide competition experiments might be used to distinguish direct sequence-specific DNA binding from the entry of Ku onto DNA from the ends. Oligonucleotides containing the MMTV GR- (Fig. 3*A*) and C3H-NRE1 elements (Fig. 3*B*), the c-*myc* plasmacytoma repressor factor binding site (PRE, Fig. 3*C*) and the NRE1 homology in the HTLV LTR (Fig. 3*D*) efficiently competed for Ku binding to NRE1-containing microcircles (summarized in Fig. 3*L*). A 100-fold excess of unlabeled oligonucleotides competed up to 90% of Ku binding to the microcircle (Fig. 3, *A–D*, *lanes 5*). The GR NRE1 element was reproducibly the most efficient competitor, again suggesting that the sequence differences between MMTV GR-NRE1 and the other NRE1-like sequences result in a reduced affinity for Ku.

By contrast oligonucleotides containing sequences from the second category of proposed Ku binding site, including the U1 promoter element (Fig. 3*E*), the heat shock element (Fig. 3*F*), the EBP80 binding site (Fig. 3*G*), and the consensus octamer motif (Fig. 3*H*), competed poorly for Ku binding to NRE1 (summarized in Fig. 3 M). Indeed, none of these sequences even competed Ku binding as well as an oligonucleotide containing a transcription factor binding site that to date has not been associated with Ku (Fig. 3, *I* and *M*), nor did they compete more efficiently than a 200-bp linear DNA fragment derived from pBluescript and known to lack sequence-specific Ku binding sites (38) (Fig. 3, *J* and *M*). These experiments indicate that category two Ku binding sites (Fig. 1) could not be distinguished from DNA end binding.

In the experiments displayed in Fig. 2, we demonstrated that one category two site, the octamer motif, appeared unable to function as a direct internal binding site for Ku on covalently closed circular DNA. To test directly whether the other category two oligonucleotides (Fig. 1*B*) might be also unable to be directly recognized by Ku in the absence of DNA ends, we assessed the ability of Ku to bind directly to covalently closed circular DNAs containing the U1, IAP, and HSE oligonucleo-

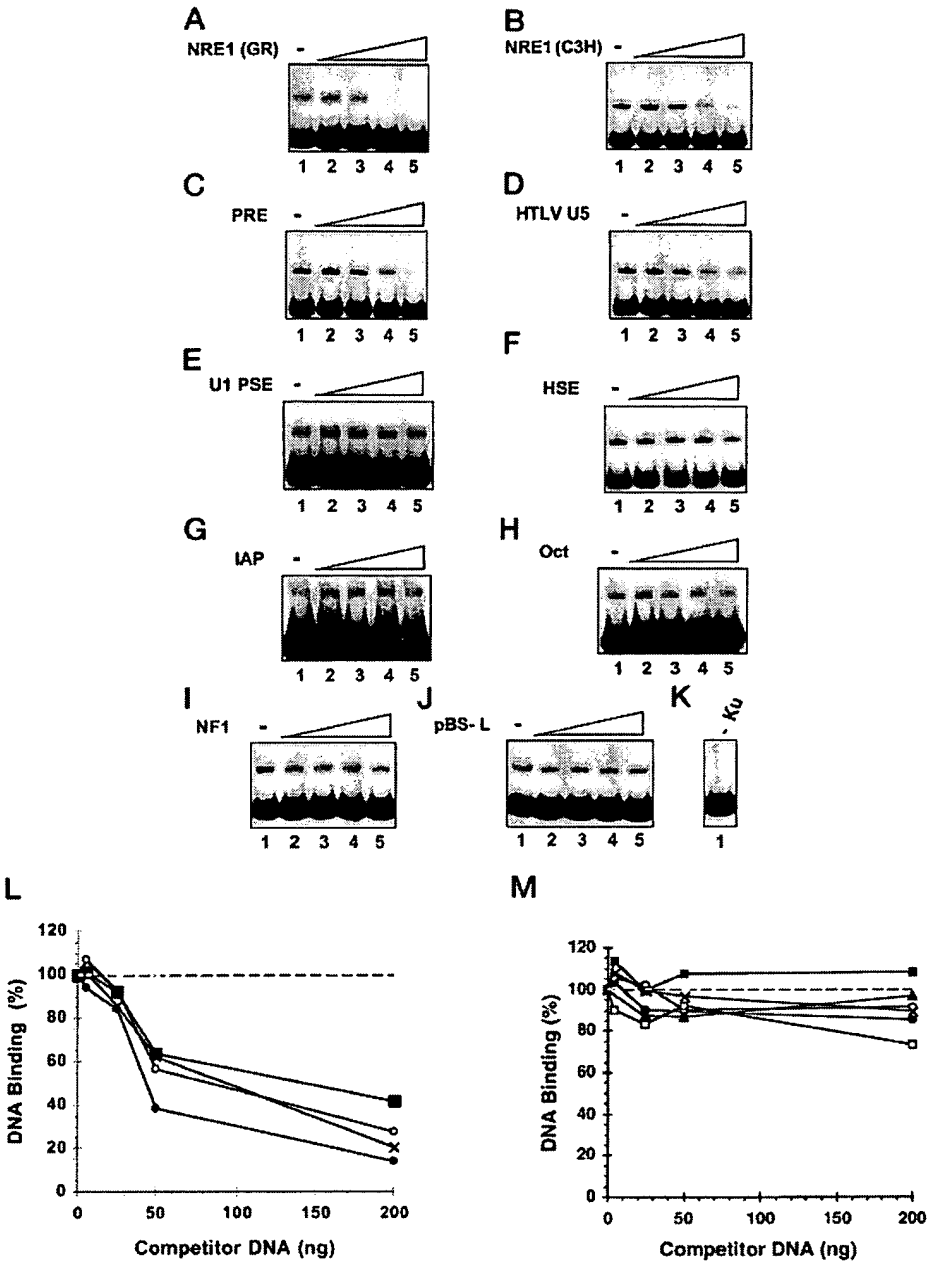

FIG. 3. **Competition of Ku binding to GR-MMTV NRE1-containing microcircles in EMSA.** *A–I*, 1 μl of recombinant Ku was incubated with ^{32}P-labeled 223-bp covalently closed circular GR-MMTV NRE1 containing microcircles prepared from pBluescript in the presence of increasing amounts of the oligonucleotides indicated. The sequences and origin of the individual oligonucleotides used are shown in Fig. 1, except for *I*, where the competition was performed with an oligonucleotide containing an NF1 recognition sequence that is described under "Materials and Methods." Amounts of competing oligonucleotides were as follows: *lanes 1*, no competitor; *lanes 2*, 5 ng; *lanes 3*, 25 ng; *lanes 4*, 50 ng; *lanes 5*, 200 ng. *J*, competition experiment performed as in *A–I* except that 25 ng (*lane 2*), 50 ng (*lane 3*), 200 ng (*lane 4*), and 500 ng (*lane 5*) of a 200-bp *XhoI/PvuII* pBluescript fragment was used as competitor. *K*, NRE1-containing microcircle electrophoresed in the absence of added Ku antigen. Each binding experiment in *A–J* was performed a minimum of three times with similar results. *L* and *M*, quantification of competition for NRE1 binding results shown in *A–J* as determined by phosphorimager. The amount of radioactivity contained in the Ku antigen-shifted complexes is plotted as a percentage of counts detected in the absence of competitor (*lanes 1*). The variation in the total number of counts loaded in each lane of each series was less than 5%. The *dotted lines* mark the amount of NRE1-microcircle binding in the absence of competitor. *L*, competition results for oligonucleotides with similarity to NRE1. Competitors were: GR-NRE1 (●), C3H-NRE1 (○), c-*myc* PRE (×), and HTLV LTR (■). *M*, competition results for oligonucleotides with no obvious similarity to NRE1. Competitors were: octamer motif (●), U1 PSE (○), HSE (×), IAP (■), NF1 binding site (□), and linear pBluescript fragment (▲). For the linear *XhoI/PvuII* pBluescript fragment, 87% of NRE1-microcircle binding remained following competition with 500 ng of DNA.

tides (Fig. 4). While NRE1-containing microcircles were readily shifted by Ku (*lanes 2* and *3*), no detectable binding was observed to the U1 (*lanes 5* and *6*), IAP (*lanes 8* and *9*), or HSE-containing microcircles (*lanes 11* and *12*).

Two conclusions can be made from these experiments. First, Ku binding to NRE1-like sequences is markedly preferred to DNA end binding. Second, the subset of proposed Ku binding sites lacking obvious homology to NRE1 did not appear to function as direct, internal, high affinity Ku DNA binding sites in our experiments. Furthermore, it appears that oligonucleo-

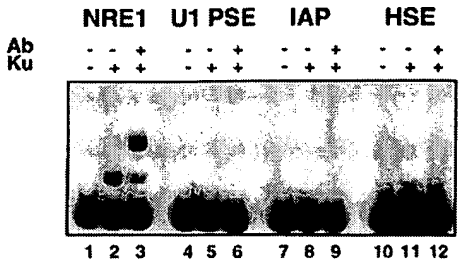

FIG. 4. **Direct binding of Ku to oligonucleotide-containing microcircles.** 1 μl of recombinant Ku was incubated with ^{32}P-labeled 223-bp covalently closed circular microcircles prepared from pBluescript containing single copies of the GR-NRE1 (*lanes 1–3*), U1 PSE (*lanes 4–6*), rc-*mos* IAP enhancer core (*lanes 7–9*), or HSE (*lanes 10–12*) in the absence (*lanes 1, 4, 7,* and *10*) or presence (*lanes 2, 5, 8, 11*) of Ku antibody Ab 162 (*lanes 3, 6, 9,* and *12*). *Lane 1* shows the covalently closed microcircles following a mock incubation in the absence of Ku.

tide competition experiments can distinguish between direct, sequence-specific Ku binding and the entry of Ku onto DNA from the DNA ends.

Affinity of Direct Binding to NRE1—To determine the affinity of direct Ku binding to NRE1, we used EMSA to perform Scatchard analyses of the binding of a constant amount of Ku to an increasing amount of GR-NRE1-containing microcircle (Fig. 5). For this assay we took advantage of the recircularization protocol for the 223-bp NRE1-containing DNA fragment that requires incorporation of a single ^{32}P label for formation of the covalently closed circular microcircle (38). Thus, the labeling efficiency of the purified microcircles used in this assay was 100%, allowing exact determination of the quantity of DNA in each incubation. A representative EMSA and Scatchard analysis is shown in Fig. 5*A*. Averaging of three independent experiments yielded a K_d of 0.84 ± 0.24 nM for direct sequence-specific DNA binding of recombinant Ku to NRE1. This value is comparable with the values obtained for the sequence-specific DNA binding of many transcription factors.

Properties of Sequence-directed Phosphorylation of GR by DNA-PK—Protein phosphorylation by DNA-PK is dependent on the tethering of the catalytic subunit of the kinase to DNA by Ku (5). While DNA-PK$_{cs}$ also contacts the DNA, it has been proposed that Ku is entirely responsible for the DNA targeting of the complex. If this is correct, DNA-PK-mediated protein phosphorylation should directly reflect Ku DNA binding preferences. To determine how the protein kinase activity of DNA-PK compared with the DNA binding preferences of Ku, we analyzed the DNA sequence preferences for phosphorylation of GR on the MMTV LTR by purified DNA-PK (65).

We first examined the conditions required for DNA-PK-mediated phosphorylation of a bacterial-expressed GST-GR fusion protein containing the DBD of GR (Fig. 6*A*). The first GST-GR protein tested contained the wild type GR DBD (*WT, lane 1*), while the second contained a Cys to Tyr mutation (C500Y, *lane 2*) in the second zinc finger of the DNA binding domain that results in a GST-GR fusion protein that is unable to bind DNA (45).

Phosphorylation of GST-GR by DNA-PK (Fig. 6*B*) was evaluated using two MMTV LTR containing plasmids as DNA templates for purified DNA-PK. pHC17 (−421/+105), contained NRE1 and the glucocorticoid-responsive element (GRE, −180/−80) from the GR-MMTV LTR (38, 46). The second plasmid, pHC364 (−364/+105), retained the viral GRE and octamer transcription factor binding sites but lacked NRE1 (38, 46). In this, and subsequent experiments, phosphorylation did not occur in the absence of any of the three components (DNA-PK, GST-GR, DNA, *lanes 1–3*). When linearized plasmids allowing Ku DNA end binding were used at the concentration

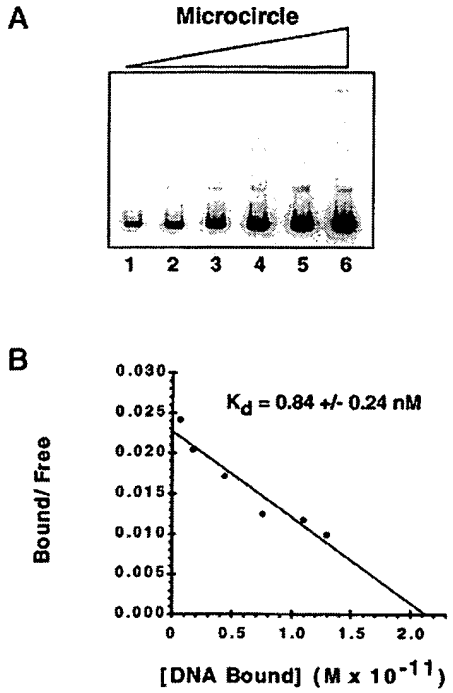

FIG. 5. **Determination of the affinity of Ku binding to NRE1.** *A*, electrophoretic mobility shift assay with increasing amounts of ^{32}P-labeled GR-MMTV NRE1-containing microcircle DNA and a constant 1-μl amount of recombinant Ku. The amounts of DNA probe added to each incubation were 2.7 pmol (*lane 1*), 5.4 pmol (*lane 2*), 10.8 pmol (*lane 3*), 21.7 pmol (*lane 5*), and 43.5 pmol (*lane 6*) as determined by scintillation counting. *B*, bound and free DNAs in *panel A* were quantified by phosphorimager and the K_d of NRE1 binding to Ku was determined by Scatchard analysis. One representative Scatchard plot is displayed together with the K_d (± S.E.) calculated from three independent repetitions of the assay.

previously described as optimal for DNA-PK-mediated phosphorylation of factors from DNA ends (5), both plasmids directed phosphorylation of GST-GR equally (*lanes 4* and *5*). As described in detail below (Fig. 8), phosphorylation occurred at a single site in the GR DBD. However, when pHC17 and pHC364 were recircularized to a covalently closed circular form resistant to nucleases ExoIII, S1, and Bal31, only the NRE1-containing plasmid (pHC17) was able to direct phosphorylation of GST-GR (*lanes 6* and *7*). Interestingly, the same results was obtained with supercoiled plasmids with the potential for localized structural transition from B form DNA (*lanes 8* and *9*). Therefore, parallel to the DNA binding activities of Ku determined above, while NRE1 efficiently directed phosphorylation of GST-GR by DNA-PK, the two octamer transcription factor binding sites in the MMTV LTR were unable to direct the phosphorylation of GR by DNA-PK.

Phosphorylation of GST-GR by DNA-PK in these experiments also appeared to require that GST-GR be bound to pHC17 in *cis* with DNA-PK. First, GST-GR$_{C500Y}$ was not phosphorylated by DNA-PK in this assay, regardless of the presence of NRE1 (Fig. 6*B*, *lanes 10* and *11*). Second, phosphorylation of GST-GR in the presence of pHC17 (Fig. 6*C*, *lane 1*) was effectively competed by addition of covalently closed circular pHC364 (*lane 3*), but was unaffected by addition of covalently closed circular pBluescript, a plasmid lacking GREs (*lane 2*).

To examine further how Ku DNA binding preferences influenced DNA-PK activity, we examined the effect of competitor DNAs on the GRE dependent phosphorylation of GST-GR (Fig. 7). In these experiments the competitor DNAs employed lacked GREs and thus were unable to compete for DNA binding of

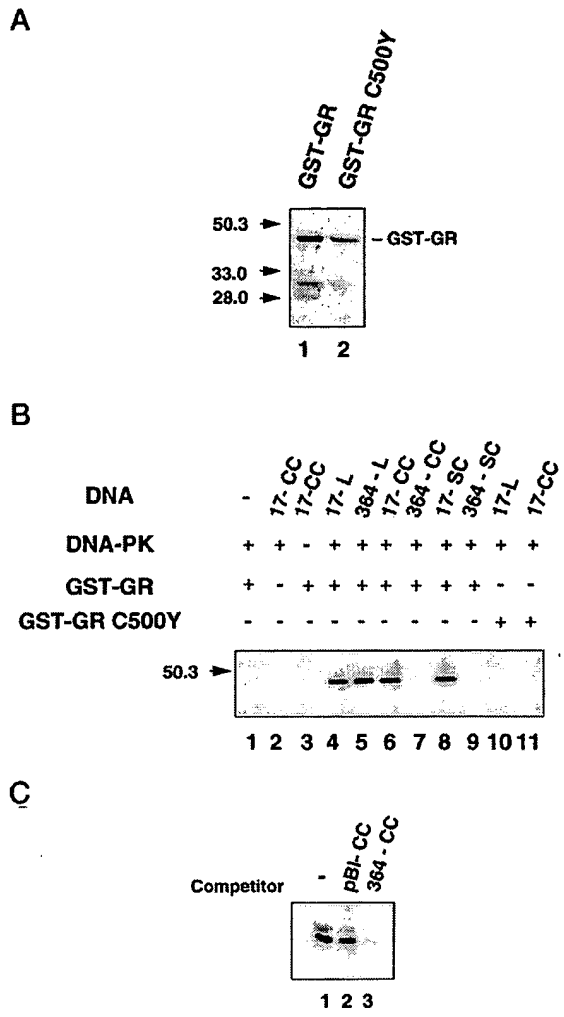

FIG. 6. **NRE1-dependent phosphorylation of recombinant GST-GR by DNA-PK *in vitro*.** *A*, silver stain of a 12% SDS-PAGE gel of slurries of 5 μl of wild type GST-GR recombinant protein (*GR*) and 15 μl of mutated GST-GR fusion protein containing a Cys-500 → Tyr substitution in the GR moiety (GST-GR$_{C500Y}$) attached to glutathione-Sepharose beads. Some preparations of GST-GR contained a second minor fusion protein band just above the 46-kDa band (see for example *panel C*). *B*, phosphorylation of rat GR recombinant protein (*GST-GR*) by DNA-PK was performed using 10 ng of the indicated DNAs and equal quantities (5 or 15 μl) of GST-GR fusion proteins and 0.5 unit of DNA-PK. The components added to each incubation are summarized above the lanes. For DNAs: *17*, pHC17; *364*, pHC364; *L*, linear; *SC*, supercoiled; *CC*, covalently closed circular; −, no added DNA. For DNA-PK: +, 0.5 unit of DNA-PK added; −, no DNA-PK added. For GR: −, 30 μl of mock *E. coli* extract slurry; +, 5 μl of affinity-purified recombinant GST-GR of rat GR expressed in *E. coli*; *GST-GR$_{C500Y}$*, 15 μl of affinity-purified mutated GST- recombinant protein unable to bind DNA. pHC17 contains both NRE1 and the GRE from the GR-MMTV LTR (−421/+105), while pHC364 is truncated 3′ to NRE1 (−364/+105). *C*, phosphorylation of GST-GR by DNA-PK on the covalently closed circular pHC17 DNA in the absence (*lane 1*) or presence of a 200-fold excess (1 μg) of covalently closed circular competing DNAs (*lane 2* and *3*). *pBl-CC*, pBluescript with no specific Ku binding site; *364-CC*, pHC364 containing GRE but no NRE1 sequence. The migration of molecular size markers (kDa) is indicated to the *left* of *panels A* and *B*.

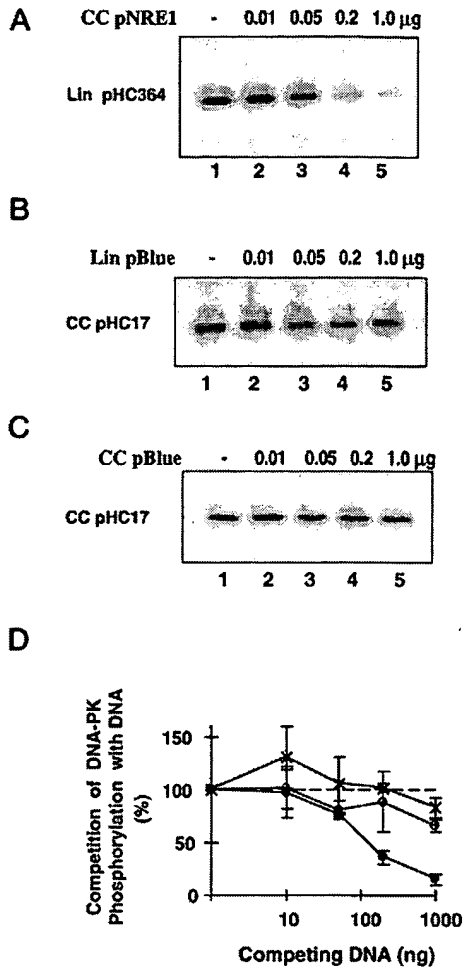

FIG. 7. **Competition of DNA-PK-mediated GST-GR phosphorylation with Ku DNA binding sites.** *A*, phosphorylation of GR was performed using 10 ng of linear pHC364 (*Lin pHC364*) in the absence (*lane 1*) or presence of the indicated amounts of the covalently closed circular pBluescript plasmid (*CC pNRE1*) containing a single copy of the GR-MMTV NRE1 oligonucleotide shown in Fig. 1. *B* and *C*, phosphorylation of GR was performed using covalently closed circular pHC17 (*CC pHC17*) in the absence (*lane 1*) or presence of the indicated amounts of linear (*B*) or covalently closed circular (*C*) pBluescript (*Lin pBlue* and *CC pBlue*) parent plasmid. *D*, quantification of the results shown in *A* (●), *B* (○), and *C* (×), as determined by phosphorimager analysis of SDS-PAGE gels. The data (± S.E.) is plotted as a percentage of the total GST-GR phosphorylation in the absence of competitor DNA. Each point is derived from three independent repetitions of the assays shown in *A–C*.

GST-GR. In the first experiment (Fig. 7, *A* and *D*), a covalently closed circular pBluescript plasmid containing a single copy of a 23-bp NRE1 oligonucleotide efficiently competed phosphorylation of GST-GR on linearized pHC364 plasmid lacking NRE1. By contrast under the same conditions, neither the linearized nor covalently closed circular pBluescript parent plasmid lacking NRE1 appreciably competed the phosphorylation of GST-GR on covalently closed circular pHC17 plasmid containing both NRE1 and the MMTV-GRE (Fig. 7, *B–D*). Thus, as for the binding of recombinant Ku to DNA, phosphorylation of DNA-bound GST-GR by purified DNA-PK displayed a marked preference for NRE1 over DNA ends.

Identification of Ser-527 of the GR DBD as a Phosphorylation Target of DNA-PK—As a first step toward characterizing the potential effects of the phosphorylation of GR by DNA-PK on the regulation of MMTV transcription by glucocorticoids (38), we sought to identify the DNA-PK phosphorylation sites on the GST-GR DBD fusion protein used in this study. GST-GR contains a single thrombin cleavage site between the GST and GR DBD moieties of the fusion protein. Following thrombin cleavage of GST-GR, the GST peptide migrated at 29 kDa, while the GR DBD migrated at 26 kDa on SDS-PAGE (Fig. 8*A*). Autoradiography of an SDS-PAGE gel containing ^{32}P-phosphorylated GST-GR cleaved with thrombin revealed a single labeled frag-

ment migrating at the position of the GR DBD (Fig. 8*B*). This radiolabeled peptide could be immunoprecipitated by a GR antibody, but not by a nonspecific control antibody.[2] Thus, DNA-PK appeared to specifically phosphorylate the DBD of the rat GR fusion protein and not the GST moiety.

Cleavage of ^{32}P-phosphorylated GST-GR with trypsin resulted in the liberation of a single radiolabeled phosphorylated peptide as determined by both reversed phase HPLC[3] and electrophoresis on a 40% alkaline PAGE gel (Fig. 9*A*). The identity of the phosphorylated GR residue was determined in two steps. First, manual Edman degradation of the tryptic phosphopeptide recovered from the polyacrylamide gel released the ^{32}P label on cycle 10 (Fig. 9*B*). As shown in Table I, tryptic digestion of the GR DBD produces three peptides with Ser or Thr at position 10. Each peptide also contains Asp, but at a different positions, suggesting that AspN, a protease that cleaves N-terminal to Asp (54) could be used to determine which peptide was the DNA-PK target.

Secondary digestion of the DNA-PK-phosphorylated [γ-^{32}P]GST-GR tryptic peptide fragment with AspN yielded a ^{32}P-labeled peptide with increased mobility on alkaline PAGE (Fig. 10*A*), confirming the presence of Asp in the tryptic peptide fragment. Edman degradation of the trypsin/AspN cleaved fragment again led to release of the ^{32}P-labeled amino acid at position 10 (Fig. 10*B*). This identified Ser-527 of the GR DBD as the DNA-PK phosphorylation site as only GR DBD tryptic peptide 518–536 would release Ser or Thr at position 10 following AspN digestion of the tryptic peptide. Furthermore, there are no comparable tryptic peptide fragments in the GST moiety of the fusion protein. Finally, the amino acid following Ser-527 in the GR DBD, Gln-528, conforms exactly to the amino acid requirements for phosphorylation by DNA-PK (Ser-Gln or Thr-Gln) (2). This is the only consensus DNA-PK phosphorylation site in the GR DBD fragment used in this study. Interestingly Ser-527 lies in the hinge region of GR that connects the DNA binding domain with the ligand binding domain and is immediately adjacent to the major nuclear localization sequence of GR (497–524) (66).

[3] J. Kwast-Welfeld, Y. Zhang, N. Weigel, and R. J. G. Haché, unpublished observation.

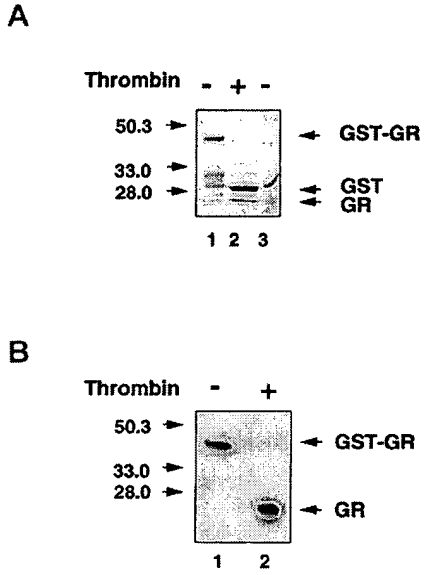

FIG. 8. **GST-GR is phosphorylated in the GR DBD.** *A*, silver-stained 12% SDS-PAGE gel of GST-GR before (*lane 1*) and after cleavage with thrombin (*lane 2*). An independent preparation of GST alone is shown in *lane 3*. The positions of GST-GR, GST, and the GR DBD alone are indicated by the *arrows* to the *right* of the gel. *B*, phosphorimager scan of GST-GR phosphorylated by DNA-PK (*lane 1*) and following thrombin cleavage (*lane 2*). For both panels the migration of molecular size markers (kDa) are indicated to the *left* of the gels.

DISCUSSION

Targeting of Ku autoantigen and DNA-PK$_{cs}$ to the LTR of MMTV appears to strongly repress the induction of viral transcription by glucocorticoids (38). In this study we have investigated the properties of sequence-specific binding of Ku to the LTR of MMTV, its effect on DNA-PK catalytic activity, and the nature of sequence-directed phosphorylation of a GR fusion protein co-localized to the MMTV promoter *in vitro*. Our results indicate that direct sequence-specific binding of Ku to NRE1-like sequences occurs with high affinity, is strongly preferred to DNA end binding, and apparently requires similarity to, but not identity with, NRE1. Several proposed sequence-specific Ku binding sites lacking similarity to NRE1 failed to bind Ku in the absence of DNA ends. Catalytic activity of the Ku-associated DNA-PK$_{cs}$ closely paralleled Ku DNA-binding site preferences. Furthermore, efficient phosphorylation of a GR fusion protein substrate required that both GR and DNA-PK be localized to the MMTV LTR in *cis*. Finally, we have identified Ser-527 in the hinge region of GR as an *in vitro* phosphorylation target of DNA-PK.

A number of sequence-specific binding sites have been proposed for Ku autoantigen (24, 30–37, 67), but to date only one site, NRE1 in the long terminal repeat of the GR-strain of MMTV, has been shown to be directly recognized by Ku in the absence of DNA ends (38). Here we have demonstrated that the related sequence in the LTR of the C3H strain of MMTV is also

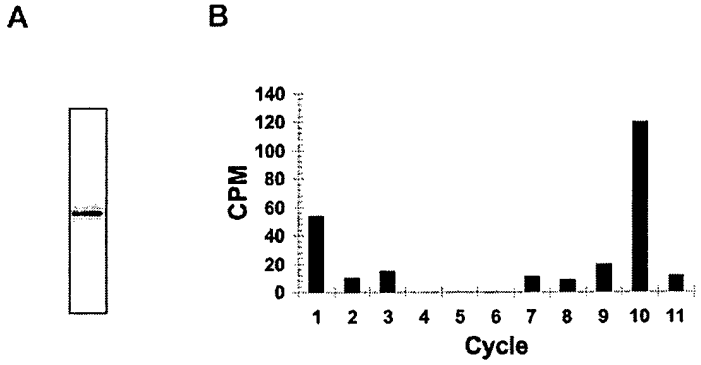

FIG. 9. **DNA-PK phosphorylates position 10 of the peptide obtained after cleavage of GST-GR with trypsin.** *A*, phosphorimage analysis of a 40% alkaline PAGE gel containing recombinant GST-GR phosphorylated by DNA-PK and digested with trypsin. *B*, plot of ^{32}P released from the phosphopeptide shown in *A* upon subsequent cycles of Edman degradation. The radioactivity released after each cycle was quantified by scintillation counting. Values were corrected for the background radioactivity (25 ± 4 dpm) of a sample blank.

a direct, sequence-specific Ku binding site, but that the sequence differences between the elements in the two strains reduce binding to the C3H motif approximately 4-fold. Interestingly, although the C3H MMTV NRE1 element was of lower affinity than the NRE1 element from the GR strain of MMTV, it was still clearly distinguishable from Ku DNA end binding in oligonucleotide competition experiments. Furthermore, the difference in affinity does not appear to affect the functionality of the C3H NRE1 element, as there is a report that sequences including the C3H NRE1 element also act to repress C3H-MMTV transcription (59).

Scatchard analysis with covalently closed microcircles containing a ^{32}P label indicated a binding constant for direct Ku binding to NRE1 of 0.84 ± 0.24 nM, comparable with the values for the DNA binding affinity of many transcription factors. Although this value is only 3-fold higher than that reported for DNA end binding of a purified Ku preparation in a similar assay (K_d = 2.4^{-9} M) with a short linear nonspecific oligonucleotide (68), our present and previous (38) competition experiments suggest that the difference in affinity between sequence-specific NRE1 binding and DNA end binding by Ku under equivalent incubation conditions is likely to be at least an order of magnitude. Additional experiments performed by an alternative assay demonstrated that the affinity of Ku for DNA ends was equivalent to its affinity for structural transitions and DNA nicks (9). Therefore, our results indicate that the GR-MMTV NRE1 element is, at least *in vitro*, the highest affinity DNA binding site for Ku identified to date.

In addition to the NRE1 motif, we evaluated Ku binding to a number of previously proposed Ku binding sites. Our results indicated two classes of sequence element. DNA elements in the LTR of HTLV and flanking the murine c-*myc* gene, which have been demonstrated to participate in repressing transcription (36, 58), have obvious sequence similarity to NRE1. These sequences competed efficiently for sequence-specific NRE1 binding in oligonucleotide competition experiments and therefore will likely prove to be direct, high affinity sequence-specific Ku binding sites. However, an important caveat to performing oligonucleotide competition experiments for analyzing Ku binding is that one must be careful to distinguish direct, sequence-specific DNA binding from DNA end binding. Therefore, conclusive evidence for direct sequence-specific binding of the c-*myc* plasmacytoma repressor factor and HTLV sequences will require that binding assays be performed with covalently closed circular templates. Nonetheless, the identification of four apparent direct, sequence-specific, Ku binding sites makes it tempting to speculate on the nature of a consensus sequence for direct sequence-specific Ku binding. Certainly, the polypurine/polypyrimidine nature of the recognition sequence is conserved in all four sites. However, experiments currently in progress indicate that the minimum length of the polypurine/polypyrimidine sequence required for direct binding may be considerably less than the 14 bp of the GR-NRE1 element.[4] Nucleotides outside the polypurine/polypyrimidine core may also play a role in Ku binding. Thus, determining exact requirements for sequence-specific Ku binding will require more extensive DNA binding analyses of the type provided by binding site selection techniques.

By contrast, the majority of the potential sequence-specific Ku binding sites proposed to date lack obvious homology to NRE1. Four representatives of this class of potential Ku binding site failed to distinguish themselves from DNA end binding in oligonucleotide competition experiments and were unable to be directly recognized by Ku in direct binding experiments with oligonucleotide-containing covalently closed microcircles. In many of the previous studies, including the octamer motif study, DNA footprinting experiments have demonstrated sequence-specific protection on linear DNA templates by Ku (32–35, 67). However in these experiments, protection was either also observed over the ends of the DNA fragments used in the footprinting experiments (34), or the footprints shown did not present the ends of the fragments employed in a manner in which DNA end binding could be evaluated (32, 33, 35, 67). Together these results indicate that accumulation of Ku over specific DNA sequences can occur in at least two ways. First, for NRE1-like sequences, Ku can directly recognize a DNA sequence element in B-form DNA. Second, Ku, which enters DNA at ends, nicks or other structural features, can, as a result of translocation, accumulate over "category two" DNA sequences.

This suggests the possibility that translocation of Ku from DNA ends is somehow sensitive to the sequence being traversed. Thus, many of the proposed sequence-specific binding sites that fail to bind Ku in the absence of DNA ends, nicks, or

TABLE I
GR-DBD tryptic peptides with Ser at position 10

The number preceding the peptide indicates the amino acid position of the first amino acid of the peptide within the rGR-DBD.

^{420}	PDVSSPPSSS CATTGAPPK
^{439}	LCLVCSDEAS GCHYGVLTC GSCK
^{518}	GIQQATAGVS QDTSENPNK

[4] W. Griffin and R. J. G. Haché, unpublished observation.

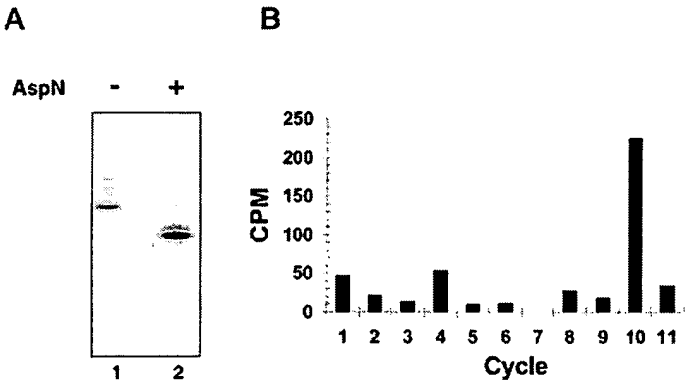

FIG. 10. **DNA-PK phosphorylates Ser-527 of the rat GR DBD.** *A*, phosphorimage analysis of a 40% alkaline PAGE gel containing recombinant GST-GR phosphorylated by DNA-PK and digested with trypsin (*lane 1*) or digested with trypsin and AspN (*lane 2*). *B*, plot of ^{32}P released from the AspN digested phosphopeptide shown in *A* upon subsequent cycles of Edman degradation. The radioactivity released after each cycle was quantified by a scintillation counting. Values were corrected for the background radioactivity (25 ± 4 dpm) of a sample blank. Ser-527 is the only possible Ser/Thr in GST-GR that can release ^{32}P at cycle 10 following both trypsin and trypsin/AspN digestion.

other relevant structural features may prove to be specific pause sites for translocating Ku that has initiated its interaction with DNA through other determinants. Our results also indicate that claims for sequence-specific Ku binding must be carefully evaluated to distinguish between direct binding and accumulation following entry onto DNA at a site remote from the sequence occupied. Furthermore, while it is apparent that the sequences unrelated to NRE1 over which Ku accumulates have the potential to regulate transcription, the lack of direct binding by Ku places additional mechanistic constraints on Ku access that must be carefully evaluated *in vivo*. The diversity in the sequences shown to accumulate Ku on linear DNA templates suggests that demonstrating, and understanding, the requirements for the accumulation of Ku over these sequences may require considerable additional investigation.

There are two alternative explanations for our DNA binding results that we cannot completely exclude at this time. First, the incubation conditions employed in this study may have precluded preferential binding to sequences unrelated to NRE1. While buffering and ionic strength in our binding assays is not discernibly different from that employed in previous studies, our experiments differed from most others in that Mg^{2+} was omitted from all binding reactions. Previously, we have shown that Mg^{2+} facilitates the translocation of Ku along DNA, which complicates the interpretation of sequence-specific Ku binding to NRE1-containing microcircles (38). Inclusion of Mg^{2+} in our binding assays however, failed to promote Ku binding to octamer motif containing microcircles or increase competition for direct binding to NRE1.[4] Second, the recombinant human Ku used in this study may lack some essential property of endogenous Ku that restricts its sequence specificity. However, in an extensive comparative analysis, we failed to determine any difference in the NRE1 binding properties of recombinant Ku and Ku purified from Jurkat T cells (38). Interestingly, Genersch *et al.* (30) recently reported that the binding of Ku to linear DNA fragments containing a transcriptional regulatory element from a collagen gene promoter required association of a Ku dimer with the TATA-binding protein (TBP). Thus, it is possible that the binding or accumulation of Ku on many of the proposed sequences unrelated to NRE1 may require the interaction of Ku with additional proteins.

One additional interesting feature of NRE1 is that, in addition to Ku, we have identified sequence specific single-stranded upper and lower-stranded NRE1 binding activities (42). Furthermore, our previous results with crude nuclear fractions and linear DNA templates suggest that nuclear factor binding to double-stranded NRE1 induces structural transitions in DNA flanking NRE1 in the presence of Mg^{2+} (69). As Ku has previously been reported to be the human DNA helicase II (70), these results suggest that Ku binding to the MMTV LTR may act to destabilize or unwind DNA around NRE1 in a manner that provides access to the single-stranded NRE1-binding factors. Although repression of MMTV transcription through NRE1 appears to require both Ku and DNA-PK$_{cs}$ (38), the nature of these additional single-stranded NRE1-binding factors and their contribution to NRE1-mediated transcriptional regulation is currently under investigation.[5]

Current models for DNA-PK-mediated phosphorylation propose that association between Ku and DNA-PK$_{cs}$ is weak in the absence of DNA (1, 5). Therefore it has been suggested that catalytically active DNA-PK is formed through the recruitment of the DNA-PK$_{cs}$ into a ternary complex in which both Ku and the DNA-PK$_{cs}$ contact DNA (5). In other instances of ternary complex formation by transcription factors on DNA, for example ternary complex factor-serum response factor-DNA assembly, specific sequences contribute to the binding of both factors to DNA (71). Thus, sequences not required for serum response factor binding to DNA alone are required for ternary complex assembly. This suggested the possibility that the DNA sequence requirements for DNA-PK activity might differ from the requirements for sequence-specific Ku binding. However, in our experiments the DNA preferences for Ku binding corresponded exactly with the preferences for the phosphorylation of GR by DNA-PK. Thus, either DNA-PK$_{cs}$, unlike ternary complex factor, does not have defined DNA sequence preferences or NRE1 contains all of the sequence information required to accommodate the DNA sequence preferences of Ku and DNA-PK$_{cs}$.

The apparent requirement for colocalization of DNA-PK and GR substrate on DNA in *cis* under our reaction conditions was striking. At the substrate concentrations used in our assays, significant phosphorylation of GR was only detected when Ku binding sites and GREs were present on the same DNA molecule. Furthermore, phosphorylation was efficiently competed by an excess of either NRE1 or GREs sufficient to titrate DNA-PK or GR onto different DNA molecules, but was refractory to competition by covalently closed circular plasmids lacking both motifs. While not an absolute requirement for substrate phosphorylation, preliminary experiments indicate that DNA sequence-dependent colocalization of GR and DNA-PK on the same DNA molecules increases the efficiency of phosphorylation of GR by at least 2 orders of magnitude.[6] These results highlight the potential importance of direct, sequence-specific DNA targeting of DNA-PK for the modulation of nuclear processes through DNA-PK-mediated Ser/Thr phosphorylation.

One of the striking features of the work on DNA-PK-mediated transcription factor phosphorylation performed to date has been that, while a large number of transcription factors are clearly DNA-PK phosphorylation targets *in vitro* (1, 27), transcription factor phosphorylation by DNA-PK in the cell remains unproven. One hypothesis suggested by our results is that DNA-PK-mediated phosphorylation of transcription factors is limited to transcriptional regulatory regions that contain binding sites for both the transcription factor and DNA-PK. Thus, it is possible that only a small proportion of a transcription factor population, those that bind to regulatory regions to which DNA-PK is also recruited, become modified. The corollary is that mutation of DNA-PK phosphorylation sites on transcription factors would be likely to result in gene specific regulatory effects.

Glucocorticoid receptor is a phosphoprotein, and the phosphorylation state of GR changes in response to binding of agonists and antagonists (72). While a number of Ser/Thr phosphorylation sites on GR have been identified (72–75), none contain the sequence requirements for modification by DNA-PK. Thus, while DNA-PK clearly has the potential to phosphorylate GR, near-stoichiometric phosphorylation of GR by DNA-PK does not appear to occur under the cell culture conditions examined to date. However, the potential importance of DNA-PK-mediated GR phosphorylation for the glucocorticoid responsiveness of genes regulated through sequence-specific Ku binding sites is evidenced by the dramatic inhibitory effect that Ku/DNA-PK$_{cs}$ have on the ability of glucocorticoids to induce MMTV transcription (38).

Our mapping of the phosphorylation of a GST-GR fusion

[5] H. Torrance, W. Giffin, and R. J. G. Haché, manuscript in preparation.

[6] H. Torrance, W. Giffin, and R. J. G. Haché, unpublished observation.

protein by DNA-PK on the MMTV LTR *in vitro* identified Ser-527 of rat GR as a potential DNA-PK phosphorylation site. Similar consensus DNA-PK recognition motifs exist in mouse and human GRs.[7] Interestingly, Ser-527 is in the exposed and apparently flexible hinge region of GR that links the DNA binding domain to the ligand binding domain (76). Thus, this serine may be accessible in the full-length GR. While not in a region of GR containing a transcriptional activation function, there is reason to suspect that phosphorylation in the hinge region of the receptor might affect transcriptional regulation. There is recent evidence that the ligand binding and DNA binding domains maintain important interactions that could be potentially modified by phosphorylation in the receptor hinge region (77). Phosphorylation in the hinge region of GR could also potentially affect receptor dimerization (78). It is also possible that phosphorylation of Ser-527 somehow modifies the potential of the immediately adjacent nuclear localization sequence of GR (79).

While this is the first report indicating the potential for GR phosphorylation in the receptor hinge region, modification of serines in the hinge region of other steroid hormone receptors has been proposed to influence the ability of these receptors to activate transcription. Substitutions of alanine for Ser-650 and Ser-530 in the hinge regions of human androgen receptor and chicken progesterone receptor, respectively, reduced the ability of these receptors to activate transcription by up to 30% (80–82).

In order to clearly resolve the potential of sequence-directed DNA-PK modification of transcription factors to influence their ability to regulate transcription, it will be necessary to first identify all of the DNA-PK phosphorylation sites on the surface of potential DNA-PK targets. Preliminary experiments with GR indicate that liganded full-length receptor can be phosphorylated at two places by DNA-PK when both are bound to the MMTV LTR.[7] Following confirmation of the identity of these two phosphorylation sites, we will be able to directly evaluate the role of modification of these residues on the ability of GR to regulate transcription.

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AGIL-117	09/710,983	Transmittal, Amendment	JSK/ BEF	
SWMC-002	10/105,774	Transmittal, Petition for a 1 Month Extension of Time *in duplicate,* Amendment After Final Rejection	JSK	$55
BEAR-010	10/360,202	Transmittal, Fee Transmittal *in duplicate,* Petition for a 2 Month Extension of Time, Amendment, Executed Declaration 1.132, Request to Change Inventorship, Statement by Inventor being Added, Consent of Assignee to Change Inventorship, Copy of Assignment, Supplemental ADS, Executed Declaration 1.76	KYB	$383
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LIFE-015	09/844,929	PTOL-85 *in duplicate*	SCT	$1,630
UCAL-168	09/848,986	Transmittal, Amendment, Exhibit 1	PAB	